IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:		§	
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	Michal AYALON-SOFFER et al	§	
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Serial No.:	10/764,833	§	
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Filed:	January 27, 2004	§	Group Art Unit: 1631
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For:	NOVEL POLYNUCLEOTIDES ENCODING SOLUBLE POLYPEPTIDES AND METHODS USING SAME	§	Attorney Docket: **27256**
		§	
Examiner:	Pablo S. Whaley	§	

Commissioner for Patents
P.O. Box 1450
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DECLARATION OF DR. MICHAL AYALON-SOFFER UNDER 37 CFR §1.132

I, Michal Ayalon-Soffer, am the Biological Experimentation Manager of Compugen Ltd. at Tel Aviv, Israel. I have a PhD in molecular biology and received my degree from Department of Cell Research and Immunology, Faculty of Life Science, Tel-Aviv University. My professional specialization is in the field of molecular biology, particularly with regard to splice variants as protein therapeutics and diagnostics. I have attached a copy of my curriculum vitae with some of the most recent publications.

I am the inventor of the present invention. I have read the present application and the new and amended claims, as well as the Office Action from the Examiner and the accompanying references. In support of the accompanying Response to this Office Action, I set forth below some of the many important differences between the present invention and the cited reference, as well as some important experimental data which demonstrates the utility and efficacy of the present invention.

In this Official action, the Examiner has rejected claims 8 and 14 under 35

U.S.C. § 101, asserting lack of utility. The results of the immunoblotting assays described hereinbelow indicate that Met-934 strongly inhibited the HGF-induced Met-phosphorylation of A549 cells. The results of the invasion assays, together with those of the scattering assays described hereinbelow indicate a strong inhibitory activity of all Met-variants on HGF-induced Met activity leading to cell motility and invasion. Further, in the urokinase assay, Met-934 inhibited HGF-induced urokinase upregulation. Taken together, these results suggest an anti-tumorigenic and anti-metastatic activity of these proteins in Met-dependent tumorigenic pathways.

The present claims are related to the protein known as Met-934, which is a splice variant of the known or "wild type" c-Met, having 934 amino acids and given in SEQ ID NO:1 of the present invention. The variant structure is described in the present application. Briefly, the variant protein of the present invention results from alternative splicing of the c-Met gene, thereby causing an extension of exon 12 (the last exon before the transmembrane region encoding exon) leading to an insertion of a stop codon and the generation of a truncated Met protein which terminates just before the transmembrane domain. It has an open reading frame (ORF) of 934 amino acids including 910 amino acids of the wild-type (w.t.) Met protein and a unique sequence of 24 amino acids at the C-terminus of the protein. It contains nearly the complete extracellular portion of Met (910 amino acids of 933 of the w.t. protein) and therefore comprises all its structural domains (the Sema, PSI and TIG domains). Met-934 is predicted to be a secreted protein since it retains the original N-terminal signal peptide (amino acids 1-24) and lacks the transmembrane domain (amino acids 933-955 of the w.t.).

As an inventor of the present application, I either performed myself or caused to be performed a number of experiments to test the utility and efficacy of Met-934. These experiments are described in greater detail below, along with graphical representation of the results. These experiments are described in separate sections below for clarity. It should be noted that the experiments were performed on Met-934 fused to Fc; however, experiments performed on a different variant of Met, Met-877, both fused to Fc and not fused to Fc, showed that the presence of Fc did not have a significant effect on the in vitro activity of the protein (data not shown).

Met-934 inhibits HGF-induced phosphorylation

Met-934 strongly inhibited the HGF-induced Met-phosphorylation of A549

cells (ATCC cat no: CCL-185), at doses equal or higher than 10nM. A similar inhibition was seen with MDA-MB-231 cells (doses equal or higher than 3nm; ATCC cat no: HTB-26). The experiments themselves are described in greater detail below.

Cells were serum starved, and Met splice variants were added prior to exposure of cells to HGF induction. A known antagonistic Fab mAb (5D5) was added in a similar manner as positive control. The cells were lysed and the phosphorylation levels of Met were determined by immunoblotting with an antibody against the specific phospho-tyrosine residues mentioned above. Blots were reprobed with a general anti-Met antibody, and the phosphorylation levels were normalized to total Met protein levels.

5D5 Fab preparation:

5D5 Fab fragments were prepared by papain digestion of mAb purified from ascites fluid. BALB/c mice were injected with 5D5.11.6 hybridoma cells purchased from ATCC (ATCC number: HB-11895). Ascites fluid was collected and antibodies were purified using Protein A.

For the generation of Fab fragments, the purified antibody was digested with papain. After dialysis, 50% papain slurry (1 ml papain coupled gel = 250 µg papain enzyme) was applied into a gravity-flow column, such that the Enzyme:Protein ratio was of 1:20 (w/w) (ie: For 2.5-3.5 mg/ml antibody use 40 µg papain). Digestion was carried out overnight at 37°C on a roller, in the presence of 20 mM Cystein-HCl.

The resulting Fab fragments were purified by anion exchange chromatography using a column of Q sepharose FF. The unbound fraction containing the Fab fragments was concentrated 50 fold and further purified by size exclusion chromatography (SEC) on HiLoad 16/60 superdex 200 prep grade column (GE healthcare, Cat# 17-1069-01). The eluted peak was pooled and concentrated 11.2 fold by a stir-cell.

The final product was analyzed for protein concentration using the Bradford protein assay with BSA standard (Bio-Rad, Cat# 500-0006) and by measurement of absorbance at 280nm wavelength. The resulting 5D5 Fab fragments were at a concentration of approximately 200µg/ml.

Cell treatments and lysis:

The following human cell lines were used: A549 (Non-Small Cell Lung Carcinoma, ATCC Cat.No. CCl-185) and MDA-MB-231 (breast carcinoma, ATCC Cat.No. HTB-26). Phosphorylation of Met in these cells lines is inducible by HGF.

Cells were seeded in 2ml growth medium (containing 10% FBS, Fetal Bovine Serum, Heat Inactivated, Biological Industries, Cat.No.04-121-1A) at 300,000 cells/well in 6-well plates. After 24hrs the cells were washed with 1ml serum free medium (0% FBS) and grown for 3 days in 2ml serum free medium. At the day of stimulation, medium was discarded and Met splice-variants, or mock were added to the cells at 3-1000nM in 250µl serum free medium, and plates were incubated at 37 °C for 1hr. As a positive control, 10nM of a known antagonistic Fab mAb (5D5) was similarly added to the cells. Subsequently, 10ng/ml HGF (R&D, Cat. No. 294-HGN) were added for 10 min (from a working stock of 10µg/ml in 0.1% BSA/PBS). The cells were washed twice with 2ml ice-cold PBS (Biological Industries, Cat. No. 02-023-5A) and 200µl of lysis buffer were added to each well: 50mM Tris pH 7.4, 1% NP-40, 2mM EDTA, 100mM NaCl, containing complete protease inhibitor cocktail (Roche, 1-873-580-001), and phosphatase inhibitor cocktails 1 and 2 (Sigma, P-2850 and P-5726). Cells were scraped with a rubber policeman and transferred to 1.5ml tubes. Lysates were incubated on ice for 30 min with occasional vortex. Lysates were centrifuged at 4oC for 10 min at 14,000 rpm, and the sup was transferred to new tubes.

Immunoblot Analysis:

Phosphorylation of Met was analyzed by immunoblotting with an antibody specific for phospho-Tyr Met residues. After stripping, the same membrane was probed again with anti-Met Ab.

Lysate samples were separated on 4-12% Bis-Tris gels (Invitrogen) in NuPAGE MOPS running (Invitrogen, NP0001). Proteins were transfered to nitrocellulose membranes using NuPAGE transfer buffer (Invitrogen, NP0006). After transfer, blots were stained with Ponceau S solution (Sigma, Cat. No. P-7170), and washed twice with TBS-T 0.1% (TBS with 0.1% Tween-20). Blocking was carried out at RT for 1hr with 5% BSA (Sigma, Cat. No. A-3059) in TBS-T 0.1%. Anti-phospho c-Met [pYpYpY1230/4/5], rabbit polyclonal Ab (Biosource, Cat. No. 44-888G) was added at 1:1000 in TBS-T 0.1% with 1% BSA, and incubated for 2hrs at RT. Blots were washed x3 in TBS-T 0.1%, and secondary Ab, peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 111-035-144) was added in blocking solution at 1:25,000, for 1hr at RT. Blots were washed x3 in TBS-T 0.1% and SuperSignal West Pico Chemiluminiscent (Pierce, Cat. No. 34080) was used for detection of HRP. Equal volumes of each solution were mixed, the blot was immersed in the solution for 5min

and exposed to film.

For reprobing with anti-Met Ab, the blot was stripped with stripping buffer (100mM beta-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.7) for 15min at 50°C, and washed x3 in PBS-T 0.05% (PBS with 0.05% Tween-20). Complete stripping was determined by re-blocking, followed by incubation with secondary antibody and detection of HRP. Blocking was carried out at RT for 1hr in 10% Tnuva milk (1% fat, Amid) in PBS-T 0.05%. Blots were washed x3 in PBS-T 0.05% prior to incubation with 1:1000 anti-Met Ab (rabbit polyclonal Ab, C-12, Santa Cruz Cat. No. SC-10) at RT for 1hr in 1% BSA, PBS-T 0.05%. Blots were washed as above, and secondary Ab, goat-anti-rabbit (see above) was added at 1:25,000 in blocking solution, for 1hr at RT. Blots were washed again, and HRP detection was carried out with SuperSignal West Pico Chemiluminiscent as described above. Autoradiograms were scanned and levels of phosphorylated Met were quantified by densitometry using ImageJ 1.36b software, and normalized to levels of Met expression.

Results:

The influence of Met-934 on HGF-induced phosphorylation of Y1230/4/5 was tested with a mAb specific to these phosphorylated tyrosine residues, as described above, using the A549 (Non-Small Cell Lung Carcinoma) and MDA-MB-231 (breast carcinoma) cell lines. Cells were exposed to 10 ng/ml HGF for 10 min, in the presence or absence of various doses of Met-inhibitory variants, as described above. Immunoblot analysis for specific phospho-tyrosines was carried out, and following stripping, the same membrane was immunoblotted with anti-Met Ab. The results are presented in Figure 1. The autoradiogram and densitometry evaluation shown in Figure 1, indicate a strong inhibitory activity of Met-934-Fc on HGF-induced Met phosphorylation in A549 cells. Figure 2 shows similar results obtained with MDA-MB-231 cells, after treatment with Met 934-Fc. In this cell line, however, also the lowest dose of 3nM seems to have a significant inhibitory effect of >60%. The conclusions from these series of experiments are as follows: Met-934 inhibits >90% of HGF-induced Met-phosphorylation in two different human cell lines, upon prior exposure to doses higher than 3-10nM of inhibitory protein.

Met-934 inhibits HGF-induced scattering

Met-934 inhibited HGF-induced cell scattering in an assay carried out with

MDCK cells.

A wide dose range was studied; the lowest dose at which inhibition could be demonstrated included 10 micrograms/ml Met-934 with 50 ng/ml HGF.

Description of cell scattering assay:

MDCK-II cells (Madin-Darby canine kidney, ECACC, Cat. No. 00062107) were used to evaluate the inhibitory effect of Met variants on HGF-induced scattering. Cells were seeded in 96-well plates at 1.5x103 cells (MDCK) per well. Cells were grown at 37°C in DMEM + 5% FBS (for MDCK). DMEM and FBS (Heat Inactivated) were purchased from Biological Industries, Cat.No. 01-055-1A, and 04-121-1A, respectively. After 24hrs, HGF (R&D, Cat.No.294-HGN) and Met splice-variants were added at various concentrations. All samples were tested in triplicates, at a final volume of 200 microl/well. At the day of induction medium was removed and 100µl assay medium containing 1 up to 100ng/ml HGF final concentration (working stock of 10µg/ml in PBS + 1% BSA) was added to all wells (except untreated control which received medium without HGF). Met splice-variants were diluted in assay medium and used at 1-100µg/ml final concentrations in 100µl assay medium. Solutions were prepared at 2X concentration, and mixed in wells at 1:1 with HGF. As controls served cells incubated with medium only, or with HGF without any inhibitors. In addition, a mock protein preparation was used as negative control. The cells were examined under microscope after 48hrs for evaluation of cell clustering and scattering. This was evaluated independently by 2 different people in the lab, in a blinded manner. A score of 1 to 5 was given to evaluate minimal up to maximal scattering activity, respectively.

Results:

Table 1 summarizes the results obtained for Met-934. As shown in the table, the lowest HGF concentration that still gave maximum cell scattering was ~ 5 ng/ml in the cell line. Met-934 clearly was able to block HGF-induced cell scattering as can be seen by the lower scores in the presence of Met-934, as compared to the high score in its absence.

Inhibitory protein	Concentration	HGF concent.	Cells	Score
Met 934-Fc	100 µg/ml	100 ng/ml	MDCK	1
Met 934-Fc	30 µg/ml	100 ng/ml	MDCK	1-2
Met 934-Fc	10 µg/ml	100 ng/ml	MDCK	5

Met 934-Fc	100 μg/ml	50 ng/ml	MDCK	1+
Met 934-Fc	30 μg/ml	50 ng/ml	MDCK	2
Met 934-Fc	10 μg/ml	50 ng/ml	MDCK	3
None	-	-	MDCK	1
None	-	5-100ng/ml	MDCK	5
None	-	3ng/ml	MDCK	3-4
None	-	1ng/ml	MDCK	1-2

Met-934 inhibits HGF-induced invasion

Met-934 inhibited HGF-induced invasion of DA3 cells, as described in greater detail below. Inhibitory activity of Met-934 on HGF-induced cell invasion was demonstrated using matrigel-coated Boyden chambers and DA3 cells, derived from a mouse mammary carcinoma.

Description of Invasion Assay:

DA3 invasion assays were performed in 96-well chemotaxis Boyden chambers (NeuroProbe, Maryland). Lower and upper wells were separated by Nucleopore filters (5 μm pore size) coated with Matrigel (3.6 μg/mm2, BD Biosciences). To test the inhibition of HGF-induced cell invasion by the Met-variants according to the present invention, the cells were treated with HGF in combination with different concentrations of Met-variants or Mock. HGF (100 ng/ml), in the absence or presence of Met-variants (at 10, 30 or 100 micro-g/ml), diluted in 30 μl DMEM+1mg/ml BSA, was placed in the lower wells. Mock was also tested at equivalent amounts to the above variant. All samples were tested in triplicates. DA3 cells (4x104) in DMEM were placed in the upper wells, and allowed to invade to lower wells by chemotaxis during a 48 hour period. Non invading cells remaining on the upper surface were removed with a cotton swab. Invading cells that migrated to the lower surface of the filter were fixed with cold methanol and stained with Giemsa. The stained filter was scanned and the area occupied by stained cells was analyzed by Photoshop.

Results of Invasion Assay:

Figure 3 shows the results of such an assay. As shown in these figures, the DA3 cells migrated through the matrigel-coated filter in response to HGF (defined as 100% migration), while very low spontaneous migration was detected in the absence of HGF. In addition, Figure 3 indicates that Met-934 strongly inhibited HGF-induced

cell invasion, at all doses, while the various Mock protein preparations did not have a significant effect.

The results of the invasion assays, together with those of the scattering assays, indicate a strong inhibitory activity of all Met-variants on HGF-induced Met activity leading to cell motility and invasion, and suggest an anti-tumorigenic and anti-metastatic activity of these proteins in Met-dependent tumorigenic pathways.

Met-934 inhibited HGF-induced urokinase upregulation

HGF stimulation in a variety of cell lines expressing Met induces the expression of the serine protease urokinase (uPA, urokinase-type plasminogen activator) and its receptor (uPAR), resulting in an increase of uPA at the cell surface. Urokinase converts plasminogen into plasmin, a serine protease with broad substrate specificity toward component of the extracellular matrix. This activity facilitates cell invasion, tumor progression and metastasis. Analysis of urokinase activity in response to HGF induction, provides a functional and quantitative assay to determine the effect of various inhibitors of the HGF/Met-mediated signaling pathway (Webb et al, Cancer Research, Vol.60, p.342-349, 2000), and can enable the assessment of the potency of Met-934.

Urokinase Assay:

Urokinase activity was tested indirectly by measuring plasmin activity, upon addition of human plasminogen and a specific plasmin chromophore (Webb et al, 2000, Cancer Res. 60: 342-349). MDCK II cells were exposed to HGF in the presence or absence of Met-934 and examined for plasmin activity after 24hrs. Percent inhibition was calculated relative to HGF-stimulated cells in the absence of inhibitor, after subtraction of background plasmin activity of unstimulated control cells.

MDCK-II cells (Madin-Darby canine kidney, ECACC, Cat.No.00062107) were seeded at 1.5x103 cells per well in 96-well plates, with DMEM + 10% FBS (Fetal bovine serum, Heat Inactivated, Biological Industries, Cat.No.04-121-1A), at a final volume of 200 micro-l/well. Cells were incubated at 37°C for 24hrs prior to induction. On the day of induction, medium was removed and 100µl assay medium containing HGF (R&D, Cat.No.294-HGN) at a final concentration of 10ng/ml (stock 10µg/ml in PBS + 1% BSA) was added to all wells (except the untreated control which received medium without HGF). Met splice-variants were diluted in assay medium and used at

medium without HGF). Met splice-variants were diluted in assay medium and used at 1 to 300nM final concentrations in 100µl assay medium. Solutions were prepared at 2X concentration, and mixed in wells at 1:1 with HGF. All samples were tested in triplicates. Wells were washed twice with DMEM without phenol red (Gibco, Cat.No.31053-028) and 200µl of reaction buffer [50% (v/v) 0.05units/ml plasminogen (Roche, Cat. No. 10874477001) in DMEM without phenol red, 40% (v/v) 50mM Tris buffer pH8.2, and 10% (v/v) 3mM Chromozyme PL (Roche, Cat. No. 10378461001) in 100mM glycine solution] were added to each well. The plate was incubated at 37°C, for 4hrs, and absorbance was measured at a single wavelength of 405 nm. Background Plasmin activity of unstimulated control cells was subtracted. Percent inhibition was calculated relative to HGF-stimulated cells in the absence of inhibitors.

Results:

Figure 4 shows strong inhibition of HGF-induced urokinase upregulation with Met-934-Fc. With doses higher than 10nM, Met-934 exhibited a strong inhibition which was higher than 90-95%. In both experiments, the Mock-Fc preparation had no effect.

As a person signing below, I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the Application or any patent issued thereon.

December 5, 2006

Michal

Dr. Michal Ayalon-Soffer

Enc.: CV of Dr. Michal Ayalon-Soffer
Figures 1-4